C-5 Propyne-Modified Oligonucleotides Penetrate the Epidermis in Psoriatic and Not Normal Human Skin After Topical Application

Paul J. White,*† Andrew C. Gray,*† Rhys D. Fogarty,† Rodney D. Sinclair,‡ Susan P. Thumiger,† George A. Werther,† and Christopher J. Wraight†

*Department of Pharmaceutical Biology & Pharmacology, Victorian College of Pharmacy, Monash University, Parkville, Victoria, Australia; †Centre for Hormone Research, Murdoch Children's Research Institute, Royal Children's Hospital, Parkville, Victoria, Australia; ‡University of Melbourne, Department of Medicine (Dermatology), St Vincent's Hospital, Fitzroy, Victoria, Australia

We have previously shown that antisense oligonucleotides effectively reduced insulin-like growth factor I receptor expression in human psoriatic skin grafted on to nude mice when injected intradermally. We therefore investigated the penetration of C-5 propyne modified antisense oligonucleotides into human normal and psoriatic skin after topical administration. Oligonucleotide (37.5 μg; 250 μM) was applied in aqueous solution or 5% methylcellulose gel for 24 h, prior to live confocal microscopy and fluorescence microscopy of fixed sections. We found that oligonucleotide could penetrate through the stratum corneum of psoriatic but not normal human skin over large regions of the epidermis. The oligonucleotide was localized to the nucleus of large parakeratotic cells in the psoriatic skin as well as smaller basal and suprabasal keratinocytes. In normal human skin, oligonucleotide was confined to the stratum corneum, with little or no oligonucleotide apparent in the viable epidermis. Electrophoresis of oligonucleotide recovered from treated psoriatic and normal skin revealed that the oligonucleotide remained intact over the 24 h period. In summary, we found that C-5 propyne modified antisense oligonucleotides could reach the target cells (in this case basal keratinocytes) after topical administration to psoriatic but not normal skin. ***Key words: antisense oligonucleotide/psoriasis/topical delivery. J Invest Dermatol 118:1003–1007, 2002***

Anti-sense oligonucleotides are increasingly being considered as therapeutic options for a range of disease states, particularly as the development of second generation oligonucleotides have reduced concerns regarding toxicity. We have recently shown that antisense oligonucleotides targeting the insulin-like growth factor-I receptor could be used to normalize the psoriatic epidermis after repeated intradermal injection into grafted human skin (Wraight *et al*, 2000). A desired feature of any cutaneous therapy is a user-friendly topical formulation, and thus further investigation of the ability of these agents to be effective, topically-applied drugs is warranted.

The use of antisense oligonucleotides in dermatology has been the subject of considerable interest due to the attractiveness of the concept of antisense therapy and the ease of access to the target organ for these large, charged therapeutic agents. The stratum corneum, however, is well equipped to prevent the access of large charged substances due to its physical, chemical, and enzymatic barrier properties. We have previously found that oligonucleotide does not penetrate normal human skin grafts on athymic mice to a significant degree (White *et al*, 1999). When grafts were tape stripped, however, there was extensive nuclear localization of oligonucleotide throughout the epidermis, indicating that the stratum corneum does act as a significant barrier to oligonucleotide delivery across the skin.

A number of studies have demonstrated antisense penetration and or efficacy using different methods to overcome the skin barrier. These methods included intradermal injection (White *et al*, 1999; Wraight *et al*, 2000), iontophoresis (Vlassov *et al*, 1994; Oldenburg *et al*, 1995; Regnier and Preat, 1998), and electroporation (Regnier and Preat, 1998). None of these methods, however, are currently well suited to use in the delivery of therapeutic agents due to considerations of cost or discomfort.

One skin condition known to involve impaired differentiation of keratinocytes and reduced stratum corneum barrier function is the common autoimmune disease psoriasis. The reduction in barrier function is attributed to a number of characteristics of psoriatic skin that are a result of the impairment of corneocyte differentiation, including impaired formation and secretion of lamellar body contents and processing of lamellar body contents into lamellar bilayers (Ghadially *et al*, 1996). Conditions in which there is an impaired ability to prevent oligonucleotide penetration may therefore be far more amenable to antisense therapy than those in which the barrier function remains intact.

The aim of this study was therefore to compare the penetration of oligonucleotides in human psoriatic skin with that in normal human skin. We found that oligonucleotide penetration in psoriasis is indeed markedly greater than that in normal skin, apparently involving the entry of oligonucleotide through areas of severe

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Reprint requests to: Dr. Paul White, Lecturer, Department of Pharmaceutical Biology & Pharmacology, Victorian College of Pharmacy, Monash University 381 Royal Parade, Parkville 3052, Victoria, Australia. Email: paul.white@vcp.monash.edu.au

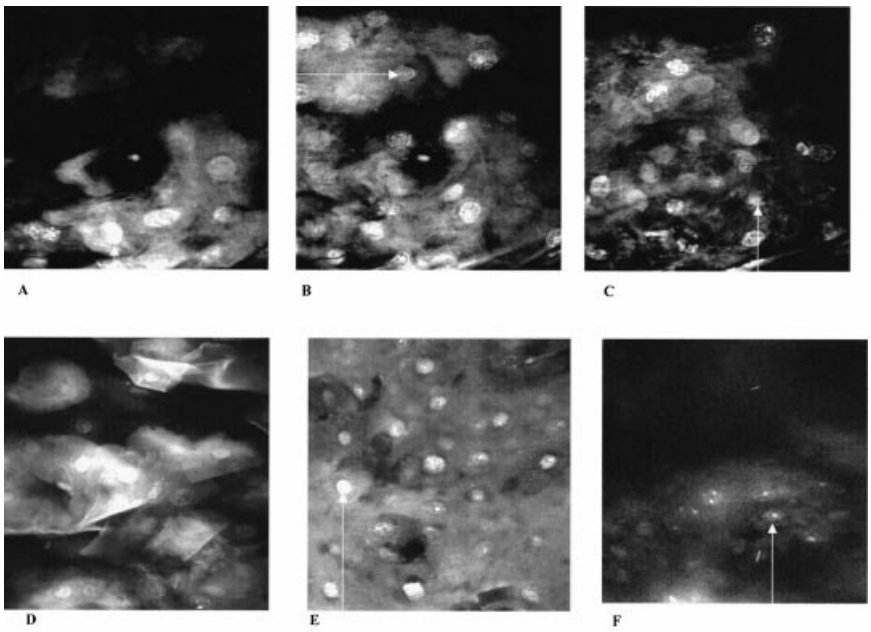

Figure 1. FITC-oligonucleotide (R451) penetrates the stratum corneum in *ex vivo* human psoriatic skin. Affected psoriatic skin was removed by punch biopsy and maintained *ex vivo* as described in *Materials and Methods*. FITC-R451 was added to biopsies for 24 h at 37°C prior to LCM. (*A–C*) FITC-R451 (37.5 μg; 250 μM) in PBS; (*D–F*) FITC-R451 (37.5 μg; 250 μM) in 5% methylcellulose gel. Nucleus indicated in (*A*) 11.7 μm, image depth = 0; nucleus indicated in (*B*) 6.4 μm, image depth = 18 μm; nucleus indicated in (*C*) 5.5 μm, image depth = 56 μm; (*D*) image depth = 5 μm; nucleus indicated in (*E*) 9.5 μm, image depth = 29 μm; nucleus indicated in (*F*)5.3 μm, image depth = 60 μm. Field of view: 100 μm.

barrier function impairment followed by lateral oligonucleotide spread throughout the viable epidermis.

MATERIALS AND METHODS

***Ex vivo* maintenance of normal and psoriatic skin biopsies** Psoriatic skin was obtained from volunteers under protocol 94012A of the Royal Children's Hospital Ethics in Human Research Committee. Two 8 mm punch biopsies were obtained from affected areas on the abdomen. Normal human skin was obtained from discards of plastic surgery. The method of skin maintenance for 24 h was adapted from that of Russo *et al* (1994). Specifically, on receipt of psoriatic or normal skin, subcutaneous tissue was removed and the skin was placed on a stainless steel mesh coated in 2% agar, with the dermis in contact with the agar. The agar was itself in contact with Dulbecco's modified Eagle's medium (Gibco BRL, Melbourne, Australia) with 10% fetal bovine serum. A reservoir containing phosphate-buffered saline (PBS) surrounded the culture medium reservoir, providing humidity.

Application of fluorescein isothiocyanate (FITC)-oligonucleotide A total of 14 normal skin samples and 13 psoriatic skin samples were used in the study. Six normal skin samples and six psoriatic skin samples received 37.5 μg (250 μM) FITC-R451 in PBS. The aqueous applications involved 3 × 10 μl applications over a period of 2 h, and imaging was performed at 24 h. Alternatively, six normal and five psoriatic samples received 10–37.5 μg FITC-oligonucleotide in 5% methylcellulose gel in a single application, with the nature of the gel imposing some variability on the final dose applied. Finally, two normal and two psoriatic samples received an alternative oligonucleotide, FITC-DT1064, in PBS as above.

Live confocal microscopy (LCM) LCM of skin samples was performed as previously described (White *et al*, 1999). The samples were imaged 24 h after oligonucleotide application for all samples. Imaging was performed using an Optiscan f900e laser scanning confocal microscope (Optiscan Ltd, Melbourne, Australia) equipped with an argon ion laser providing 488 nm (blue) excitation and with fluorescence detection above 515 nm (green). For each sample, a series of horizontal sections were taken at increasing depths into the epidermis. Pilot experiments demonstrated that fluorescence could be detected up to 100 μm below the skin surface.

Assessment of oligonucleotide penetration in fixed sections of human skin After live confocal imaging, the skin was fixed in 4% paraformaldehyde for 24 h followed by 24–72 h in 0.5 M sucrose. The samples were then embedded in paraffin wax, and 5 μm sections were cut and mounted on silanized slides. Sections were dewaxed in histolene, rehydrated in graded ethanols, rinsed, and mounted with Mowiol (Calbiochem, Clayton, Victoria, Australia) with DABCO anti-fade (Sigma, St Louis, MO).

Analysis of oligonucleotide stability The treated skin samples were homogenized with a Kinematica PT 1200 Polytron in 20 mM Tris–HCl pH 8.0; 150 mM NaCl; 10% glycerol buffer containing 1% Triton X-100, and centrifuged at 20,000 × *g* for 10 min at 4°C. The pellet was resuspended in distilled water containing 1 mg per ml proteinase K and incubated for 1 h at 56°C. Formamide (25 μl) was added and the sample heated to 55°C for 5 min The samples were analyzed on a 19% polyacrylamide 90 mM Tris borate, 20 mM ethylenediamine tetraacetic acid gel, containing 7 M urea and transferred on to a Zeta-Probe membrane (Bio-Rad, Sydney, Australia). The membrane was blocked with 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20 containing 5% skim milk powder for 1 h at room temperature, then incubated with alkaline phosphatase-conjugated anti-fluorescein immunoglobulin (Vistra ECF Western blotting kit; Amersham, Buckinghamshire, U.K.); 1:2500 dilution for 1 h at room temperature. Bands were visualized by chemifluorescence.

Oligonucleotides Two different-3′-FITC-conjugated C-5 propynyl dC dU phosphorothioate oligonucleotide 15mers were used, with the following sequences: R451—UAACACGAUACGCGA; DT1064—CACAGUUGCUGCAAG.

RESULTS

FITC-oligonucleotide penetrates the stratum corneum of human psoriatic skin after topical administration in PBS or 5% methylcellulose gel LCM Imaging of skin treated with FITC-R451 as described in *Materials and Methods* showed clear penetration of the oligonucleotide into subsurface keratinocytes over the entire surface of the skin when in aqueous solution (**Fig 1*A–C***) or in 5% methylcellulose gel (**Fig 1*D–F***). **Figure 1** shows oligonucleotide localized in large (10–15 μm diameter) parakeratotic cells at the surface of the skin (**Fig 1*A***) or orthokeratotic cells just below the surface of the skin (**Fig 1*D***, optical section 5 μm below the surface), which were nucleated and very thin as determined by optical sectioning. As imaging continued deeper into the skin, smaller oligonucleotide-containing nuclei were apparent, with the smallest cells observed to contain nuclei characterized in a previous study to be basal/suprabasal (≈ 5 μm diameter; White *et al*, 1999). **Figures 1(*B*, *C*)** and (***E***, ***F***) show images obtained from optical sections 18–60 μm below the surface of the skin after treatment with oligonucleotide in PBS or methylcellulose gel, respectively, showing keratinocyte nuclei had taken up oligonucleotide at this depth.

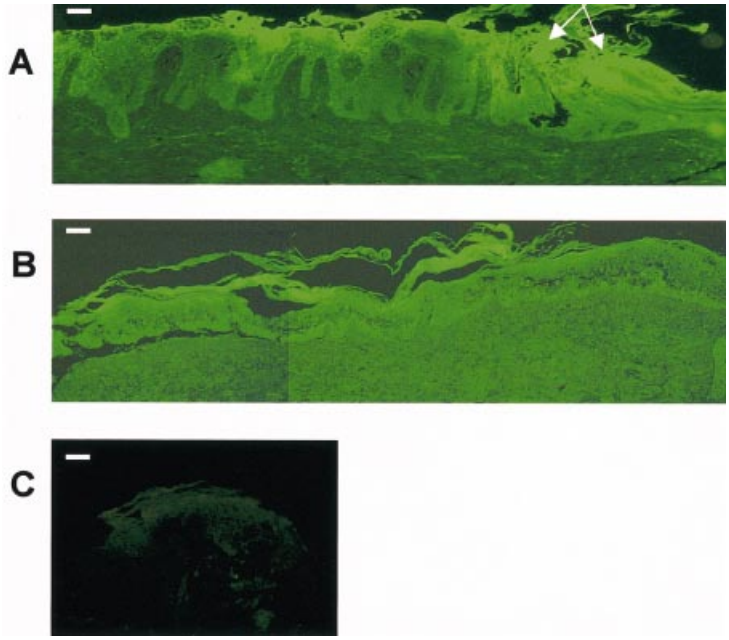

Figure 2. Fluorescence microscopy of fixed sections confirms that FITC-oligonucleotide (R451) penetrates the stratum corneum in *ex vivo* human psoriatic skin. Affected psoriatic skin was removed by punch biopsy and maintained *ex vivo* as described in *Materials and Methods*. FITC-R451 was added to biopsies for 24 h at 37°C prior to fixation (4% paraformaldehyde), embedding and sectioning. (*A*) FITC-R451 (37.5 μg; 250 μM) in PBS; (*B*) FITC-R451 (37.5 μg; 250 μM) in 5% methylcellulose gel; (*C*) control no FITC-oligonucleotide sections for fluorescence. *Scale bars:* (*A*, *B*) 100 μm; (*C*) 50 μm.

Fluorescence microscopy of FITC-oligonucleotide distribution Topical oligonucleotide-treated psoriatic skin samples were also imaged using fluorescent microscopy after fixation and sectioning. **Figure 2** shows representative fluorescence imaging of sections treated with FITC-R451 in PBS (**Fig 2*A***) or 5% methylcellulose (**Fig 2*B***). Oligonucleotide appeared to penetrate to the basal epidermis over a wide area of the biopsy for each of the donors. Arrows in **Fig 2**(***A***) show apparent points of entry through the stratum corneum, from which the oligonucleotide may have gained access to a wide area of the epidermis.

The pattern of widespread distribution and nuclear localization was consistent with minor variability across the biopsies from the different patients. Also, an alternative FITC-labeled oligonucleotide (DT1064) exhibited similar distribution characteristics to the oligonucleotide used for the majority of the experiments (R451).

FITC-oligonucleotide does not penetrate the stratum corneum of normal human skin after topical administration in 5% methylcellulose gel or PBS Both LCM and fluorescence microscopy of fixed sections demonstrated that oligonucleotide does not penetrate through the stratum corneum of normal skin samples. **Figure 3**(***A***, ***B***) show LCM images of skin that received FITC-R451 in 5% methylcellulose and PBS, respectively. Consistent images of fields of large squamous cells were obtained from all normal skin samples imaged after topical application. The diameter (often 20–30 μm), lack of thickness (often < 10 μm) of the cells and the lack of apparent nuclei were indicative of fully differentiated stratum corneum acting as a barrier for the oligonucleotide. Optical sections taken deeper into the epidermis revealed no fluorescence (data not shown), indicating a lack of penetration of the oligonucleotide into normal skin. This was confirmed by fluorescence microscopy of fixed sections of the samples imaged using LCM. **Figure 3**(***C***) shows a fluorescence microscopy image of oligonucleotide appearing at the surface of the epidermis and not any deeper into the skin. This was consistent for each of the samples examined and was confirmed by immunohistochemical analysis of the FITC-oligonucleotide location (data not shown).

Figure 3(***D***) shows an image of normal human skin not treated with oligonucleotide, demonstrating that no autofluorescence was detected using the same confocal settings as images in **Figs 3**(***A***, ***B***).

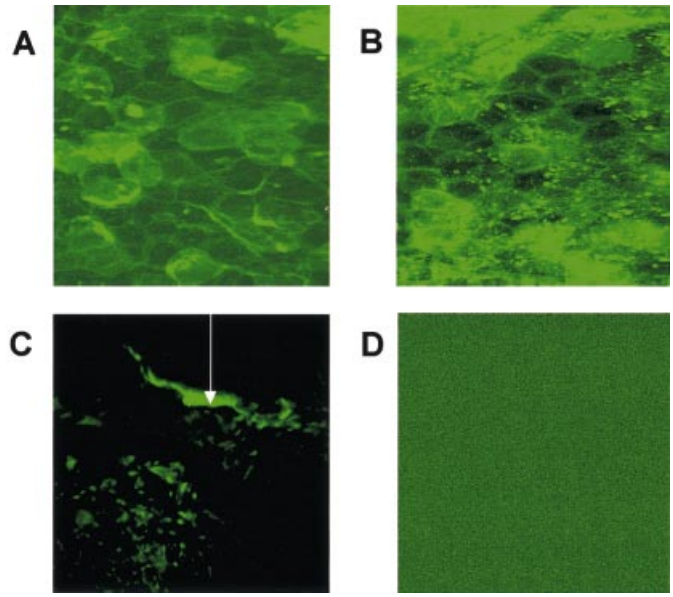

Figure 3. LCM and fluorescence microscopy of fixed sections demonstrate that oligonucleotide does not penetrate the stratum corneum of normal human skin. Normal human skin was maintained *ex vivo* as described in *Materials and Methods*. FITC-R451 was added to biopsies for 24 h at 37°C prior to LCM and finally fluorescence imaging of fixed sections. (*A*, *B*) LCM of FITC-R451 (37.5 μg; 250 μM) in 5% methylcellulose gel; (*B*) FITC-R451 (37.5 μg; 250 μM) in PBS; (*C*) fluorescence microscopy of FITC-R451 (37.5 μg; 250 μM) in 5% methylcellulose gel (*arrow* indicates stratum corneum); (*D*) image of control (no FITC-oligonucleotide) confocal image of normal human skin. *Scale bars*: (*A*, *B*) 20 μm; (*C*, *D*) 100 μm.

FITC-oligonucleotide remains intact after topical administration in both normal and psoriatic human skin There was no apparent degradation of the oligonucleotide after topical application for either normal or psoriatic skin. **Figure 4** shows a western immunoblot of homogenized samples of psoriatic skin from four individual donors treated with FITC-R451 in 5%

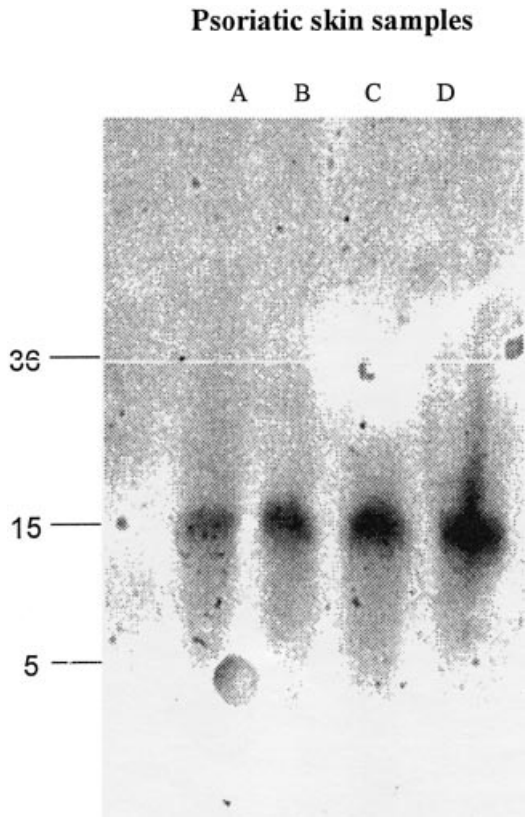

Figure 4. Oligonucleotide stability after topical application to skin maintained under organ culture conditions. Samples of normal and psoriatic skin treated with 37.5 μg (250 μM) FITC-DT1064 in 5% methylcellulose were homogenized and analysis of oligonucleotide size was performed as described in *Materials and Methods*. Homogenate samples were analyzed by western immunoblot following denaturing polyacrylamide gel electrophoresis. Each of the samples from psoriatic skin (samples A–D) appeared to contain full length (15mer) oligonucleotides as evidenced by a single band that appears at the same position as the 15mer control sample.

methylcellulose. Analysis of oligonucleotide size was performed as described in *Materials and Methods*. Each of the samples appeared to contain full length (15mer) oligonucleotide as evidenced by a single band that appears at the same position as the 15mer control sample.

DISCUSSION

The results of this study indicate that oligonucleotide in aqueous solution or methylcellulose gel can penetrate the stratum corneum of psoriatic skin maintained *ex vivo* from a range of individual donors. LCM indicated that oligonucleotide can be detected in the nuclei of small, undifferentiated keratinocytes, over the entire area of application of the oligonucleotide. The pattern of uptake was similar in skin receiving oligonucleotide in aqueous solution or methylcellulose gel, although brighter fluorescence was detected in the skin that received oligonucleotide in aqueous solution. Fluorescence imaging of fixed sections confirmed the LCM finding that oligonucleotide does penetrate throughout the epidermis. Significantly, local areas of severely impaired stratum corneum formation appeared to allow extensive oligonucleotide entry to the basal epidermis, from which lateral spread of the oligonucleotide appeared to occur. Oligonucleotide did not appear to penetrate through regions of the stratum corneum that are relatively intact and properly formed. These findings were not dependent on oligonucleotide sequence, as an alternative FITC-labeled oligonucleotide exhibited similar distribution characteristics to the oligonucleotide used for the majority of the experiments. Normal skin maintained in the same *ex vivo* apparatus demonstrated its ability to prevent the penetration of oligonucleotide through the stratum corneum. No fluorescence was found below the stratum corneum in 14 skin samples maintained *ex vivo* with oligonucleotide in aqueous solution or methylcellulose. The lack of penetration of oligonucleotide across normal stratum corneum was in agreement with the findings of Butler *et al* (1997) but in contrast to those of Wingens *et al* (1999) and Mehta *et al* (2000). Wingens *et al* (1999) used chimeric oligonucleotides that could have different pharmacokinetic properties to those oligonucleotides used in this study. Mehta and colleagues found that phosphorothioate oligonucleotide could cross the stratum corneum of human skin grafts after application of a 2% oligonucleotide cream. The variance in results could be as a result of the oligonucleotide dose (we used ≈ 1/20th of the oligonucleotide dose of that used by Mehta *et al*, 2000) or varying limits of detection. The problem with the use of large amounts of oligonucleotide is the increased likelihood of nonsequence-specific effects of the oligonucleotide. It was found previously that repeated intradermal injection of 2.5 μg oligonucleotide produced a reliable antisense effect but also a small nonsequence-specific effect (Wraight *et al*, 2000. The use of greater oligonucleotide dose may allow for some penetration of oligonucleotide into the viable epidermis of human skin, but may result in significant nonsequence-specific antisense effects. In any case, at the doses used in this study, oligonucleotide was found to penetrate the stratum corneum of psoriatic human skin and not normal human skin.

The oligonucleotide used in this study was intact after topical delivery to both normal and psoriatic human skin. A clear single band was apparent after electrophoresis of the recovered oligonucleotide, and the band was at the same position as a 15mer control sample. This result is somewhat surprising as oligonucleotide systemically delivered is quickly broken down by nucleases (Crooke *et al*, 1996) and the epidermis has a large amount of enzymatic activity. These results are, however, consistent with those of Vlassov *et al* (1994), who found intact oligonucleotide in mouse tumors after topical administration.

C-5 propyne-modified oligonucleotides were used in this study. These modified oligonucleotides were selected because their increased affinity for target mRNA allows inhibition with lower concentrations (Wagner *et al*, 1993) and shorter oligonucleotide length (Flanagan *et al*, 1996) than unmodified phosphorothioates, theoretically reducing the incidence of aptameric effects on target cells. One of the aims of this study was to deliver these oligonucleotides topically into psoriatic skin, and therefore the same chemical modifications were used in this study.

The results of this study indicate that oligonucleotides can be topically applied to psoriatic skin in simple topical formulations and efficiently reach the basal epidermis fully intact. This suggests that antisense therapy is particularly suited to the treatment of psoriasis and that complex topical formulations may not be required. The issue of penetration of oligonucleotide in normal skin is somewhat more complex. This study indicates that phosphorothioate oligonucleotide does not penetrate a wide range of normal human skin samples to any significant degree.

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